Effect of anti-CIRP antibody on inflammatory response, tumor formation and abdominal aortic aneurysm in rats

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Abstract

**Purpose:** To investigate the effect of anti-cold induced RNA binding protein (CIRP) antibody on inflammation, tumor formation and abdominal aortic aneurysm in rats.

**Methods:** Thirty healthy male Wistar rats were assigned to pseudo-operation, abdominal aortic aneurysm model, and anti-CIRP groups, with 10 in each group. The levels of CIRP, TNF-α, monocyte giant cytokine chemokine-1 (MCP-1), Toll-like receptor 4 (TLR4), and nuclear factor kappaB (NF-κB) were determined compared among the groups.

**Results:** At both 2 and 4 weeks, the expression of CIRP protein in the model group was significantly higher than that in the sham operation group (p < 0.05). At these two time-points, tumor formation and maximum diameter were higher in anti-CIRP and model control rats than in pseudo-operation rats. After 4 weeks of treatment, the protein expressions of TNF-α, MCP-1, TLR4, and NF-κB were higher in anti-CIRP and model control rats than in pseudo-operation rats, but were lower than model control values (p < 0.05).

**Conclusion:** CIRP expression is significantly increased in abdominal aortic aneurysm tissue and serum, and is involved in the onset and progress of abdominal aortic aneurysm. Anti-CIRP antibody therapy effectively suppresses tumorigenesis, and inhibits tumor wall inflammatory reaction via TLR4/NF-κB pathway. This finding provides a clue and new strategy for the clinical management of abdominal aortic aneurysm.

**Keywords:** CIRP, Abdominal aortic tumor wall, Inflammatory reaction, Protein expression, Tumor body

INTRODUCTION

Abdominal aortic aneurysm refers to an arterial degenerative disease in which the abdominal aortic diameter is increased by > 50% [1]. Abdominal aortic aneurysm is more common in elderly men, especially in smokers. It has been reported that smoking leads to a significant increase in the risk of aneurysm rupture [2]. In addition, atherosclerosis is a risk factor for abdominal aortic aneurysm [3]. Most patients with abdominal aortic aneurysm have no obvious symptoms and signs. Patients with obvious symptoms usually undergo surgical treatment. The most common symptom prior to rupture of the tumor is pain [4].
high mortality. The incidence of abdominal aortic aneurysm has gradually increased in China, due to aging population, and it has become one of the common diseases in the elderly population [5]. It has been reported that inflammatory reaction in abdominal aortic tumor wall is important in the formation, progression and rupture of abdominal aortic tumor wall. It releases a large number of inflammatory factors such as TNF-α and monocyte giant cell chemokines-1 (MCP-1), resulting in damage to the tumor wall. Moreover, chemotactic inflammatory cell infiltration and release of a large number of matrix metalloproteinases lead to aggravation of tumor wall damage [6]. Therefore, inflammatory reaction in the abdominal aortic tumor wall is linked to the pathogenesis of abdominal aortic tumor.

It has been reported that the expression of cold-induced RNA binding protein (CIRP) is low in normal tissues and cells, but it is increased in many stress states [7]. Clinical studies have shown that CIRP is released outside the cell, and it is involved in inflammatory injury in many diseases [8]. The present study investigated the effect of anti-C1RP antibody on inflammatory reaction, tumor formation and abdominal aortic aneurysm in a rat model of abdominal aortic aneurysm.

EXPERIMENTAL

Animals and grouping

A total of 30 healthy male Wistar rats aged 15 weeks and weighing (180 ± 50) g, were housed 10 rats per cage and fed adaptively in an environment with room temperature of 19 °C and 55 %. They were allowed ad libitum access to feed and water, and a fluorescent lamp was used every 12 h for illumination.

This research was approved by the Animal Ethical Committee of Department of General Surgery, Tianjin 5th Central Hospital, Tianjin, PR China (approval no. 201935598), and carried out according to Principles of Laboratory Animal Care [9].

The rats were randomly divided into sham operation group (n = 10) and abdominal aortic aneurysm model group (n = 20). Rat abdominal aortic aneurysm model was established through elastic protease perfusion. The abdominal aortic aneurism rats were randomly divided into model control group (without immunoactive IgG treatment) and anti-CIRP (antibody CIRP antibody therapy) group, with 10 rats in each group. At 1 week, 2 weeks and 4 weeks after treatment, samples were taken and tested.

Reagents and instrument

The reagents and instruments used, and their sources (in brackets) were: sheep anti-CIRP antibody (Wuhan Boote Biotechnology Co. Ltd.), mouse anti-actin antibody (Xiamen Research Biotechnology Co. Ltd.); second antibody kit (Baao de Biotechnology Co. Ltd. produces); HRP labeled second antibody (Beijing Borxi Technology Co. Ltd. produces); DAPI staining solution (Wuhan Seville Biotechnology Co. Ltd.); RT-PCR related kit (Jinlong Biotechnology Co. Ltd.); fetal bovine serum (Zhejiang Tianhang Biotechnology Co. Ltd.); 1640 medium (Shanghai Junrui Biotechnology Co. Ltd.); double antibody (penicillin/streptomycin; Hefei Bome Biotechnology Co. Ltd.); and mouse anti-MCP-1, TNF-α antibody (Puptek Biotechnology Co. Ltd.).

Others were surgical instruments (Shanghai Jinzhong Surgical Instruments Factory); Bsupermachine (produced by Xuzhou Dawei Electronic equipment Co., Ltd.); Cell incubator (Jiangsu Ruiming Biotechnology Co. Ltd.); super-clean worktable (Yisgao Trading Co. Ltd.); fluorescent microscope (Leica Microsystem Trading Co. Ltd.); ordinary Optical microscope (Beijing Optic Technology Co. Ltd.); Cell culture bottles and cell culture dish (Grena Biotechnology Co. Ltd.), and centrifugal tube (Tianjin Bensheng Health Technology Co. Ltd.).

Protein expression analysis

Protein expressions of CIRP, TNF-α, MCP-1, Toll-like receptor (TLR4) and Nuclear transcription factor kappa B (NF-κB) in tumor wall tissues of rats in each group were assayed with western blotting: First, the tumor wall tissues of each group were collected, and the total cell protein was extracted by adding protein lysate, and the BCA kit was used to determine and quantify the protein concentration. Then, 10μL protein samples were added to equal volume of loading buffer, mixed and boiled at 100 °C until protein denaturation. The denatured protein was transferred to PVDF membrane for 1h after 10 % SDS-PAGE. The membrane was blocked with 5 % skim milk powder at 37°C for 90 min. Antibodies for CIRP, TNF-α, MCP-1 and Toll antibody were 1:500 ratio and incubated with the membrane overnight at 4°C, incubation with secondary antibody for 60 min at 37 °C. Finally, electrochemiluminescence was used to develop the protein bands.
Polymerase chain reaction

The RT-PCR method was used to determine CIRP mRNA expression in the tumor wall of rats. The tumor wall tissues of rats in each group were taken and the total RNA was extracted according to the RNA extraction instructions. The concentration of the extracted miRNA was determined using ultraviolet spectrophotometry. The extracted RNA was reverse-transcribed to cRNA in accordance with the protocol of Invitrogen’s reverse transcription kit. The CIRP mRNA expression level was determined with RT-PCR, with β-actin as internal control. The RT-PCR reaction conditions were: pre-denaturation at 95°C for 3 min, 95°C for 5 sec, 60°C for 30 sec, in a total of 40 cycles. The CIRP mRNA expression was analyzed with 2^△△CT formula.

Ultrasound imaging assessment

Ultrasound was used to detect changes in the tumor bodies and the maximum diameter of tumor in each group. The rats were put under continuous anesthesia using an anesthetic mask. The thorax hair was removed from the anesthetized rats on the operating table. The left lateral position was maintained, and the limbs were fixed. The tumor was examined with a high-frequency linear array superficial probe (Biosound SL2325, 6MHz-19MHz). Appropriate coupling agent was applied to the probe, and the probe was placed at the level of the left pectoral papilla muscle. A 2-d ultrasound depth was adjusted to 1.5-2.0 cm to obtain the maximum diameter of each tumor. After the examination, the images obtained were preserved.

Statistical analysis

Data for the expression of CIRP, extent of tumor and maximum diameter of tumor are expressed as mean ± standard deviation (SD). Statistical analysis was done with SPSS 19.0. Statistical significance was assumed at p < 0.05.

RESULTS

CIRP expressions in rat tumor wall tissues and in serum

At 1 week, 2 weeks and 4 weeks, CIRP mRNA level was markedly higher in model rats than in pseudo-operated rats, and the expression was time-dependent. At weeks 2 and 4, CIRP protein expression in model rats was significantly higher than that in the sham rats (p < 0.05).

Table 1: CIRP expressions in rat tumor wall tissues and serum

<table>
<thead>
<tr>
<th>Group</th>
<th>Time</th>
<th>Sham group</th>
<th>Model group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 week</td>
<td>2 weeks</td>
<td>4 weeks</td>
</tr>
<tr>
<td>CIRP mRNA</td>
<td>1.00 ± 0.23</td>
<td>2.56 ± 1.09a</td>
<td>6.78 ± 1.23a</td>
</tr>
<tr>
<td>CIRP protein</td>
<td>2.99 ± 0.24</td>
<td>5.43 ± 1.52a</td>
<td>5.93 ± 1.45a</td>
</tr>
<tr>
<td>β-actin</td>
<td>0.67 ± 0.78</td>
<td>1.52 ± 1.45a</td>
<td></td>
</tr>
</tbody>
</table>

aP < 0.05, compared with the sham group

Effect of anti-CIRP antibody therapy on abdominal aortic aneurysm in rats

At weeks 1, 2 and 4, tumor formation and maximum tumor diameter were markedly higher in model control rats and anti-CIRP rats than the corresponding values in pseudo-operation rats. Moreover, the maximum tumor diameter in the anti-CIRP group was significantly lower than that in the model control group (p < 0.05). At weeks 2 and 4, tumor formation in the anti-CIRP group was significantly lower than that in the model control group (p < 0.05). These results are shown in Table 2.

Table 2: Effect of anti-CIRP antibody therapy on abdominal aortic aneurysm in rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Time/ week</th>
<th>Tumor formation rate (%)</th>
<th>Maximum tumor diameter (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>1</td>
<td>0.00</td>
<td>1.23 ± 0.11</td>
</tr>
<tr>
<td>Model control</td>
<td>1</td>
<td>67.11a</td>
<td>1.64 ± 0.43a</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>83.23a</td>
<td>1.93 ± 0.69a</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>100.00a</td>
<td>2.45 ± 0.68a</td>
</tr>
<tr>
<td>Anti-CIRP</td>
<td>1</td>
<td>66.99ab</td>
<td>1.45 ± 0.29ab</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>67.00ab</td>
<td>1.69 ± 0.44ab</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>82.63ab</td>
<td>1.75 ± 0.57ab</td>
</tr>
</tbody>
</table>

aP < 0.05, vs sham operation rats; bP < 0.05, vs model control rats

Effect of anti-CIRP antibody treatment on inflammatory response in abdominal aortic aneurysm in rats

After 4 weeks, the expression levels of TNF-α, MCP-1, TLR4 and NF-κB were markedly higher in anti-CIRP and model control rats than in sham-operation rats, and they were markedly reduced anti-CIRP rats, relative to model control rats. These results are presented in Table 3 and Figure 2.

DISCUSSION

Due to advances in vascular molecular biology, the molecular mechanism involved in the pathogenesis of abdominal aortic aneurysm are now better understood.
Table 3: Effect of anti-CIRP antibody treatment on expressions of TNF-α, MCP-1, TLR4 and NF-κB in rats with abdominal aortic aneurysm

<table>
<thead>
<tr>
<th>Group</th>
<th>Expression of TNF-α</th>
<th>Expression of MCP-1</th>
<th>Expression of TLR4</th>
<th>Expression of NF-κB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>0.25 ± 0.13</td>
<td>0.42 ± 0.16</td>
<td>0.22 ± 0.13</td>
<td>0.15 ± 0.06</td>
</tr>
<tr>
<td>Model control</td>
<td>0.78 ± 0.17&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.14 ± 0.15&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.59 ± 0.15&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.56 ± 0.09&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Anti-CIRP</td>
<td>0.51 ± 0.11&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.71 ± 0.13&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.47 ± 0.15&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.28 ± 0.10&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>P < 0.05, vs sham rats; <sup>b</sup>p < 0.05, vs model control rats

Figure 1: Effect of anti-CIRP antibody treatment on expressions of TNF-α, MCP-1, TLR4 and NF-κB in abdominal aortic aneurysm of rats

On the basis of these molecular mechanisms, drugs for the treatment of abdominal aortic aneurysm have been developed and applied in clinical practice. These drugs focus mainly on inhibiting arteriosclerosis and tumor wall inflammation [8]. At present, many drugs are used for treating abdominal aortic aneurysm. One of these drugs is angiotensin II receptor antagonist which inhibits abdominal aortic aneurysm by hindering inflammatory reaction in the tumor wall. Indeed, anti-inflammatory drugs are still the mainstream treatment for abdominal aortic aneurysm. Related studies have confirmed that antibiotics and non-steroidal anti-inflammatory drugs significantly suppress abdominal aortic aneurysm [10].

Cold-induced RNA binding protein (CIRP) is an endogenous inflammatory factor which up-regulates the expression of TNF-α and other inflammatory mediators, leading to inflammatory waterfall reaction and aggravation of tissue damage [11]. Other studies have shown that CIRP is highly expressed in many tissues and organs, and CIRP-targeted treatments significantly reduce tissue injury [12,13]. The TLR4 is an endogenous immune recognition receptor which is involved in inflammatory response of many diseases. Some studies have shown that TLR4 is overexpressed in abdominal aortic aneurysm, and knockout of TLR4 significantly suppresses abdominal aortic aneurysm. The levels of MCP-1 and other inflammatory factors in the tumor wall were significantly decreased [14]. One important target in the treatment of chronic inflammation is NF-kappa B. Previous studies have shown that NF-kappa B is overexpressed in abdominal aortic aneurysm. Inhibition of the expression of NF-κ B and downstream TNF-α suppress the development of abdominal aortic aneurysm [15].

In this study, a rat model of abdominal aortic aneurysm was established using elastic protease perfusion, and the expression of CIRP mRNA in tumor wall tissue and serum expression of CIRP protein was determined with RT-PCR and immunoblotting, respectively. The results showed that the expression of C1RP in abdominal aortic aneurysm wall and serum was significantly increased. In addition, the effect of anti-C1RP antibody therapy on inflammatory reaction, tumor formation and abdominal aortic aneurysm in rats was studied. The results showed that % tumor formation and maximum diameter were markedly higher in anti-CIRP and model control rats than in pseudo-operation rats, and the maximum diameter and % tumor formation in the anti-CIRP group were significantly lower than those of the model control group.

These results suggest that anti-CIRP antibody therapy significantly suppresses abdominal aortic aneurysm, indicating that C1RP may play an important role in the pathogenesis of abdominal aortic aneurysm wall. Moreover, the protein expressions of TNF-α, MCP-1, TLR4, NF-κ B were markedly elevated in anti-CIRP and model control rats, relative to the corresponding expressions in sham-operated rats. However, the expressions were appreciably lower in the anti-CIRP rats than in the model control rats. This indicates that anti-CIRP significantly inhibits inflammatory reaction in abdominal aortic artery and the chemotactic effect of tumor inflammatory wall on cells. These are beneficial for reduction of the degree of infiltration of inflammatory cells in the tumor wall. Thus, the inhibitory effect on inflammatory response of tumor wall may be achieved through suppression of the activation of the TLR4/NF-κappa B pathway and down-regulation of TNF-α expression.

CONCLUSION

Abdominal aortic aneurysm is associated with significantly increased expressions of CIRP in abdominal aortic aneurysm tissue and serum.
Anti-CIRP antibody therapy effectively hinders formation of the tumor, and inhibits inflammatory reaction in the tumor wall through a mechanism associated with the TLR4/NF-κappa B pathway. These findings provide a new insight and new target for the clinical management of abdominal aortic aneurysm.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the author(s) named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. All authors read and approved the manuscript for publication. Yuqing Wang conceived and designed the study. Yuqing Wang, Lantao Lu, Weiyans Li, Shuntong Gu collected and analysed the data. Yuqing Wang wrote the manuscript.

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